General and Comparative Endocrinology 283 (2019) 113229

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Identification of neuropeptides in the sea cucumber *Holothuria leucospilota*

Hoang Dinh Chieu[a,b], Saowaros Suwansa-ard[a], Tianfang Wang[a], Abigail Elizur[a], Scott F. Cummins[a,*]

[a] Genecology Research Centre, University of the Sunshine Coast, 90 Sippy Downs Drive, Sippy Downs, Queensland 4556, Australia
[b] Research Institute for Marine Fisheries (RIMF), 224 LeLai Street, HaiPhong City, Viet Nam

A B S T R A C T

Neuropeptides play important roles in the regulation of physiological processes such as growth, metabolism and reproduction. In sea cucumbers (Phylum Echinodermata), numerous neuropeptides have been identified and some are attributed to reproductive processes. In this study, our goal was to gain a better understanding of the neuropeptide repertoire for the black sea cucumber *Holothuria leucospilota,* a species that has been severely overfished from the wild due to human consumption. We applied *in silico* transcriptome analysis of the adult *H. leucospilota* radial nerve cord, gonad and body wall to elucidate 35 neuropeptides that are conserved throughout the Bilateria. Then, liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of radial nerve cord was employed and showed an additional 8 putative novel neuropeptide precursors, whose predicative cleaved peptides do not share sequence similarity with any reported neuropeptides. These data provide an important basis for experimental approaches to manipulate *H. leucospilota* broodstock reproduction and growth in culture, which will hopefully re-establish population numbers.

1. Introduction

Neuropeptides are intercellular signalling molecules secreted by neurons and acting as hormones, neurotransmitters, or modulators. They play important roles in regulating physiological processes such as growth, metabolism and reproduction in the animal kingdom (Burbach, 2011; Hökfelt et al., 2000; Nässel and Larhammar, 2013). Neuropeptides are derived from neuropeptide precursors which are processed into bioactive peptides (Nässel and Larhammar, 2013). The role of neuropeptides in mediating invertebrate physiological processes has been studied most comprehensively within arthropod (e.g. insect and crustacean) and molluscan models (Zhang et al., 2018; John and Lynne, 2017). The echinoderm phyla are of special interest for studies in comparative endocrinology because of their phylogenetic position in the animal kingdom as deuterostomian invertebrates and their pentaradial symmetry, which is a unique context for analysis of the physiological and behavioural roles of neuropeptide signalling systems (Rowe et al., 2014).

Identification of neuropeptides is the first step to unravel their bioactive function(s). Nowadays, neuropeptide precursors can be identified through genome and transcriptome data or in combination with mass spectrometry (MS). The first extensive analysis of neuropeptide signalling systems in an echinoderm was enabled by the sequencing of the sea urchin (*Strongylocentrotus purpuratus*) genome (Burke et al., 2006; Sodergren et al., 2006), which led to the identification of 20 neuropeptide precursors (Rowe and Elphick, 2012). Subsequent analysis of other echinoderm transcriptomes including the starfish *Asterias rubens* (Semmens et al., 2016), *Acanthaster planci* (Smith et al., 2017) and sea cucumbers *Apostichopus japonicus* (Rowe et al., 2014), *Holothuria scabra* (Suwansa-ard et al., 2018) and *Holothuria glaberrima* (Mashanov et al., 2014), allowed the identification of 17–48 neuropeptide precursors. The human genome contains around 90 genes that encode neuropeptide precursors, thus other echinoderm neuropeptides may still be present.

Sea cucumbers are widely considered as commercially valuable with a high demand for consumption and use in some traditional medicines (Olivera-Castillo et al., 2013; Fahmy et al., 2015). The black sea cucumber *H. leucospilota*, has a very high level of protein (43.23–48.27%) and carbohydrates (44.62–48.56%), as well as the lowest level of total lipids (4.6%) of all sea cucumbers tested (Nahla, 2013). Thus, this species represents an excellent alternative choice for people who prefer low fat diets. As a result, it has been overfished from the wild, which has led to the rapid exhaustion of wild populations (Lovatelli, 2004; Purcell et al., 2011). Knowledge of the neuropeptide repertoire in *H. leucospilota* will enable for the future functional studies and a better understanding of the regulation of physiological and developmental processes (e.g. growth, feeding, reproduction, and behavior). In combination with biotechnology, this will facilitate the improvement of breeding and seed production in this species aquaculture.

In this study, we generated a transcriptomic resource using next-generation sequencing of *H. leucospilota* radial nerve cord (RNC), gonad and body wall. Using this transcriptome sequence data, we have

* Corresponding author.
E-mail address: scummins@usc.edu.au (S.F. Cummins).

https://doi.org/10.1016/j.ygcen.2019.113229
Received 8 October 2018; Received in revised form 17 July 2019; Accepted 22 July 2019
Available online 23 July 2019
0016-6480/ © 2019 Published by Elsevier Inc.

identified 43 neuropeptide precursors through *in silico* prediction and mass spectrometry. Some putative novel neuropeptide precursors were also found that are present in other echinoderms.

2. Material and methods

2.1. Animal and sample collection

H. leucospilota were collected from Port Cartwright, Mooloolaba, QLD, Australia and housed in a protein skimmed saltwater aquarium system at the University of the Sunshine Coast (Sippy Downs, QLD, Australia). During dissection, the whole body wall was kept on ice, then a sample for each tissue (RNC, body wall, and gonad) of both males and females at the immature (stage 1) and fully mature gonad stage (stage 4) was isolated and immediately frozen in liquid nitrogen before storage at −80 °C until used. Classification of gonad maturation stages was based on previous reports (Fujiwara et al., 2010a; Morgan and Neal, 2012; Gaudron et al., 2008; Drumm and Loneragan, 2005).

2.2. RNA extraction, transcriptome analysis and assembly

RNA extraction of RNC, body wall (male and female) and gonad (ovary and testis) tissues was performed using the TRIzol reagent (Life Technologies, Australia). RNA quality and concentration were checked by BioAnalyser 2100 (Agilent Technologies, Australia). Five cDNA library constructions were prepared at the University of the Sunshine Coast (USC) including: (1) radial nerve cord; (2) female body wall; (3) male body wall; (4) ovary and (5) testis. Each library construction was combined by both immature and mature female/male stages. These library constructions were prepared by the SENSE mRNA-seq library prep Kit V2 (Lexogen, Austria), then sequenced using an Illumina HiSeq2000 instrument (Illumina Inc.) at the Australian Genome Research Facility Ltd. (Australia). The details of samples used for reference libraries are presented in Supplementary Table S1. Trimmed reads from Illumina sequencing corresponding five cDNA library constructions above were used for *de novo* assembly using the CLC Genomic workbench software version 10.1.1 (CLC Bio-Qaigen, Aarhus A/S) with parameters set as follows: seqType, fq; minimum k-mer coverage = 4; minimum contig length of 100 bp; group pair distance = 250. The raw sequences of the transcriptome dataset were submitted to the NCBI Sequence Read Archive (SRA) database under the Genbank accession number PRJNA510690.

2.3. In silico identification of neuropeptide precursors in Holothuria leucospilota

Sequences of neuropeptide precursors identified previously in other echinoderm species were used as the input for sequence-based similarity searches (BLASTp). These included neuropeptide precursor proteins from sea cucumbers *H. scabra* (Suwansa-ard et al., 2018), *H. glaberrima* (Mashanov et al., 2014), *A. japonicus* (Rowe et al., 2014), starfish *A. planci* (Smith et al., 2017), *A. rubens* (Semmens et al., 2016) and sea urchin *S. purpuratus* (Rowe and Elphick, 2012). The open reading frames retrieved from BLAST searches were screened for the presence of signal peptide sequences using the SignalP 4.0 (Petersen et al., 2011) and for neuropeptide features such as recurrent basic residue cleavage sites (KK; KR; RK; RR) using Neuropred (Southey et al., 2009).

2.4. Identification of neuropeptides from radial nerve cord of Holothuria leucospilota using mass spectrometry (MS)

RNCs were excised from mature female sea cucumbers and radial nerve extract (RNE) was prepared as described in our previous study (Chieu et al., 2018). RNE was filtered through an Ultrafree-MC, HV 0.45 µm pore-size sterile filter (Millipore Corporation, Billerica, MA, USA), then centrifuged at 15,000 RPM for 20 min at 4 °C. The supernatant was conditioned with TFA (final concentration 0.1% TFA) then separated by ultra high-pressure liquid chromatography (uHPLC) using a C18 column with 2.7 µm particle size – Advancebio peptide Map (Agilent, USA). The uHPLC fractions (5 min/fraction) were collected and digested by trypsin (Promega, Australia) in-solution using the method described previously (Ni et al., 2018). Then, trypsin-digested peptides were resuspended in 100 µL 0.5% formic acid in MilliQ water and analysed by LC-MS/MS on an ExionLC liquid chromatography system (AB SCIEX) coupled to a QTOF X500R mass spectrometer (AB SCIEX) equipped with an electrospray ion source. Twenty microliteres of each sample were injected onto a 100 mm × 1.7 µm Aeris PEPTIDE XB-C18 100 uHPLC column (Phenomenex, Australia) equipped with a SecurityGuard column for mass spectrometry analysis. For mobile phases, solvent A consisted of 0.1% formic acid (aq) and solvent B contained 100% acetonitrile/0.1% formic acid. Linear gradients of 5–35% solvent B over 10 min at 400 µL/min flow rate, followed by a steeper gradient from 35% to 80% solvent B in 2 min and 80% to 95% solvent B in 1 min were used for peptide elution. Solvent B was held at 95% for 1 min for washing the column and returned to 5% solvent B for equilibration prior to the next sample injection. The ionspray voltage was set to 5500 V, declustering potential (DP) 100 V, curtain gas flow 30 psi, ion source gas 1 (GS1) 40 psi, ion source gas 2 (GS2) 50 psi and spray temperature at 450 °C. The mass spectrometer acquired mass spectral data in an Information Dependent Acquisition mode. Full scan TOF-MS data was acquired over the mass range 350–1400 and for product ion ms/ms 50–1800. Ions observed in the TOF-MS scan exceeding a threshold of 100 cps and a charge state of +2 to +5 were set to trigger the acquisition of product ion. The data was acquired and processed using SCIEX OS software (AB SCIEX, Concord, Canada). The MS/MS data was searched against *H. leucospilota* transcriptome-derived protein database using PEAKS studio (BSI, Waterloo, Canada). Identified proteins that did not match to any known neuropeptide precursors were further screened for the presence of signal sequences and cleavage sites, as described above.

2.5. Comparative sequence analysis of novel neuropeptides

The putative novel neuropeptide precursors were identified as the open reading frames for which no previously identified neuropeptide precursors were matched in BLAST searches. Then, the presence of *H. leucospilota* novel proteins were investigated in the genome of *A. japonicus* (Zhang et al., 2017) and transcriptomes of *H. scabra* (Suwansa-ard et al., 2018) and *H. glaberrima* (Mashanov et al., 2014) using BLAST search, and subsequently used for comparative sequence analysis. Sequence alignment and similarity were analyzed by using the MEGA7 software (Kumar et al., 2016).

3. Results and discussion

3.1. In silico identification of Holothuria leucospilota neuropeptides

Illumina sequencing led to a combined total of 131,509,682 clean reads from the five *H. leucospilota* tissues. A *de novo* assembly resulted in 258,477 contigs with average contig length of 471 base pairs (bp) and an N50 of 435 bp, which includes 105,733,235 matched reads. An in-depth summary of the combined *H. leucospilota* transcriptome library, assembly and quantification is presented in Supplementary Table S2.

By *in silico* BLAST search, 35*H. leucospilota* homologs of neuropeptides that have been characterised previously in six other echinoderms (*H. scabra, H. glaberrima*, *A. japonicus*, *A. planci, A. rubens*, and *S. purpuratus*) were identified. Among them, 33 full-length neuropeptide precursors were found as determined by the presence of a start codon, a predicted N-terminal signal peptide, and a stop codon (Fig. 1 and Table 1). Only the thyrotropin-releasing hormone-like (TRH-like) and NP30 peptide precursors were determined to be partial-length due to

RGP (Relaxin-like gonad-stimulating peptide)

MASKTIRVVFFAAVCVLLVLEEAASTRLCGRELSRAIYRICSHGKRGYPMVDLEEEDFSQELDTEWDEFLAQALTGLLESRTFAADIESDRYFTIPQRFRRSGGIARRCCASGCSSSDIAKLC*

Cubifrin (NGIWYamide)

MAVEAKIVFFSLVVCIWFASFVTCQTITGRPQDYEEISKAVDRFLDILVKEDTTDPDFKLDSWETLLDEEINPKLKILAHVMRSLSSRPDIPSVREQLYIPTSYLQPFITDEDAYSNDPSSKLPSLQS SGLDQIKNKERNNYWGENDIFPENEEKRNGIWYGKRATNTDDAATKRNGIWYGKRNSPTIEDKRNGIWYGKRNGIWYGKRNGIWYGKRDSGYYPSVADEVM*

GPB5-like 1 (Glycoprotein hormone beta-5-like 1)

MARRERVYLLGLTGCLIVSLLCSTVTGVDPSTTIECFVHTAMKHRAVKNGCRPYDINIRGCWGRCDSYQVPELLPPYVYSVHPSCIQDSVTPTTIQLPDCDEGVDSTYVYESANSCKCQSISDVTTA YDYRPDYYLT*

GPB5-like 2 (Glycoprotein hormone beta-5-like 2)

MEQTSSRLAVFIFVVLNILAIKSTSASPAPIFRGLPLSCRVREYTEYKAEKPGCRPQRIVVDACYGFCDTYQVPALRAPYKLSQHKMCSYGETVEVSIQLDDCDSGVDRTFTYINARNCVCRKCT PENTFCLGIH*

Luqin-like

MALSRCRTTEIFCWCAILLILAALMVPLSSAQSMEASRKRKPYKFMRWGKRFYDDTDVLEVDSSEDLGFEQQQLPTELLLPLFGDKEIICMKVSEGGLYQCSQYTARSDTLRHK*

Myomodulin-like

MALPSSFLCIAVTVLGVCHLTVSQPLDSEEVSNFQAEVPDDLETAEILFQNEKNLAHQLALDYLYDGEEANDFTNFAKRDPRNEEMDKKDTRTLYDNYARRNIPPNFIKLNFGKRTTDKRFNSPF IFGKREDLSEVDKKAYSPFLYGKRERDLDLQPDKXXQTGLQQVVLWKTRKQGQGWL*

Corazonin-like 1

MNFPSVTGKAAVALLLIVIFSVACQAHNTYSMKGKYRWRAGKRSYNFRDSLEKKMPSYSNMFQGIQQYPLQDDVPPSSKSLKSIVTDLRNYCDSLLQLLDELDVAIPENKSE*

Corazonin-like 2

MGRNSVAATLFLAVVFGSILCSAHNTFSMSGQNRWKAGGKRSDPTVGRPQQNFLDPSSYSSDQPGENTITLREMLIDMRDYCNFLLKLLDNARLAQTERK*

PDF-type (Pigment-dispersing factor-type)

MSQLTLLAVCGVVLLLAAHTYCTDDHREKRIGDNDYFQAKYNDAQENQRQRARWSLLNEKMRNAGKRSGVNRDFEDDLYRNMEREFIRRLIGEYFYQLEESP*

CRH-type (Corticotropin-releasing hormone-type)**

MNDIHRLILLLSLGTIALLLCLPACSDAQPVGKFQFDLDELPEAGLEDWSWKPPPLVLTRQRVLERLADLAMSRSGSGPGYTLPRKRVASGVGLRFFPLDRVAMDEQLRNDWDLLARMERNQ ETFEDIGRRR*

Calcitonin-type

MKPTAVLTLAVFCTFYTIATAASLSRDAADTVFPLKGDELRDLADKVDAYNQIVHTFSRSSEFQSMLKRSSSGCAEYFGGCAQLKLGQDALSRMLADSNSRFGSGGPGKRRRSVDAVAEDKA*

Orcokinin-type

MPGVGAKTPRALRTWLLFGVICWVFASSSRAEIISDEAETREEELNKLALEELREDLVDELLEDLVERAQRMLRGPPNSNLINLVSGWHSSGRQKKGLFGAQQLTHVKSGFHKKNIDDDGLLG AYAEDKRGRSVFGSRLTNLNSGFHKRGGEDKREGEEFKRGRALFSHQLTNLASGFTKKSDESDLFQEDKRGRTGFSGASRLTNLNSGFSKRNDGQEEVKRGRASFSSGLTNLASGFTKRDD EKTSLFDEDKRGRSAFSGASRLTNIGSGFKRGGNEGPWEEGLEKRGRSAFSGASRLTHIGSGFKRSDDEGRWKESKDKRGRTAFSAARSLTNLDSGFKRDGNFNTGPMVHIPSGFVEKRSV ETPSKESL*

Thyrostimulin2-like (Thyrostimulin alpha-2 subunit-like)

MRLRVTVTYLAHIAAILLFQCTAADQWNPRAGCHLVGYQKEVRIPGCHTAWVRMNACRGYCMTYSFLSDSATLERSGGTQLFTSHGSCCVITSTHDVHITLQCENNQVYKDTFKSARTCNCALCAIP*

Bursicon-like

MEPSHPSTNTSATVMVKCALIALLLSGLISQCSATCRRQALIHLIDHEGCTLRRVPSFGCRGTCTSYSRVSPTDYTRMERSCQCCQESDHMMRGVRLNCPELFPSTKVVEVKVAMSCTCRPCHGGSGV PSETRLEDLLQ*

AN-type (AN peptide-type precursor)

MARFEVMLLLMSVLVAVTVLQCRADEENTADEDQVKQVLQEIYDEPDEDKRANRYRTLNRRPGNRRVIKSDPAMQEEEGEEANAEEKRGNRFRTVGGRPRNRMMRVTKSDPEAESGENLE ANDEEKRANRFRTLGGRPRNRMMRVTKSDPGEEVEENLEANDEEKRGNRFRTVGGRPRNRMMRVTKSDPGEEVEEIGRASCRERG*

TRH-like (Thyrotropin-releasing hormone-like)

MSTLTFLLCFIYLLQGNVVRGTPEGTIEAVEGEILREIEDNAQLNEISPGGEDEKRQYFAGKRQLPGGDEYEDKRQYFAGKRQLPGGDAGDLEDKRQYFAGKRQLPGGDAGDFEDKRQYFAG KRQLPGGDEYQDKRQY*

Myoactive-like (MPMNPADYFSRGTVYIPTRDS precursor)

MHLHVITRVTLLVVCMYMCIAAVSSVPIDLTDYLTRGTVYFPTRDRRDAEDGSDDYDTRGTVWFPSQKRRSSPFPGFGDYFTRGTVYLPSRDRRNYVPVEPGMMDHMCVIKPSFYCMCTANG YVSFCENHTSSYFTGK*

Orexin-like

MMRLQWYILQIVLVIILATCLSLTNAQRGCCSRVVDCNIPRECFCPLKKSMCKDGARRHFISGKRSDINWNYHPAEDDWNEFKYLLNEDRETDRPLNSQTITRTLIRRLGVDPDLLITAFQEGNSKY GSKKYYEES*

Holotocin-like

MKMSLKYVCGAVLFLILVVCLRESRSCFVTNCLLGGKRSASRPYRQCLPCGPRQSGRCVGPGICCGNSFGCLINTKETITCRRENELPTPCEVIGDRCLTVSNGKCTAFGVCCNERGCVLEEN CKYSPSRMRNEPLLMTSSNENLYDGGIGERFTDFLFEESEK*

NP11a

MLRATILALFAVLLVSDLVVRALPVDSGADTFLNELLNQNLMERRSHKQTGQCVSNCVSCSKYVGMVADRCIRGCIQKPSMKGAINNAEVDAWTACEKFLYR*

NP11b

MNTFLVCLLSTILLSGVVLGAPTFDFGDDANYDAILDERMRRSYHKINECLHDCLFCAKHSKKFNIRACMDQCGGKVTGNEQTWAACSMFTGRK*

NP15a

MKAVMCFCALALLFEAVIALPYADADDEFDARDMAKRVVDILSPASHHLDKRASSLCPMDCFSCFKFIRDLTPDPCVKGCQRRHMVGDASYSYDRKMWNRCAQYLQRPLAR*

NP15b

MSQLTSLCLLLIVAAAAVFAYPAEQGVNLLDLDAIFDAAEPEDEGEIQEVAPNSFDRERRRQNYSCMRECSHCAKIHTRYSMVRCFRTCSSGRTDFGCNNRMHFV*

NP18a

MHSSTAVTLATCLIIATIVTQSATALAFQGSQDRAKRLFWVDKKSANGDSTVCVRASAADDIAECFISECIKRQLDCEMICDRQDCHSLCQTKKLDCAKACLTENSANNVLSQ*

NP18b

MNMSNLIYLAAFATVSAFLMHTVSSISYNTIDGTIPYKRLFWIDKKSSYDDSSLYTVPISSAPSIVECFLDDCTKNFATCAKSCIMTNEEIPCMTACKQQRTVCSIRCFQRYAMKSSDNAA*

NP21**

MCWTALICSMLLASFLSKNCPANSPVSSGTLQSSFLVPMSERRGNSALFMSWIVASSRALCWKRRGRRLKVNSMSNLSPLTNLSISSLEKPRTTSSISTRWT*

NP23

MNLTLKLLLLFVTFCIFIIWSVESVELVDATADEDATDEEQNLIDLDEYPLMTLLEKLVASRQRDTRSWRSNPKFRHYKAQSAGWKRGPMSDDEAHLVDQRRWNSGSRYNGYACQGNRCSAGWKRT PGVAAYEDLS*

NP25**

MAVVPGKTTHGLVWLLLFLVISAVCLQSCHAARRCSVKGCMVHFGKRIAPFSEEDEEPGTEYIKAQDYQEQQRQKNLQILRLVDRLLSGSMTSRNFPLDDETE*

NP26**

MSTKTFMLVVCLAYTLLLVTALPLDYPSDENNAFLRRLRSNKSPYCTYGGLTCICDKNNKPKSCGLVYYDPRWNI*

NP27**

MHRFIAICLALLVLAATSLAFPAQLEYLLNEDPDEQVAKRDTDEDAMYRVQRSRDSCSINCIACGSTWSTKFNTHQCMKHCQTITSPDSPKKYSSCRRFYIN*

NP28**

MTGTTATKMCVLLATFVVLLATLTEMANTMDLPYSEDENLPFGVMERPGGSHGVWQRRTVDILLEALTLLREEMDKPMGQDAQQVVKKGNSHKQPLSVLEDTGD*

NP30**

MAGGITLVGSYIRQVHGRGHQWSFHTLLVCLAACVLATQVMRCKGQCADSDRPIIMNTYRQHLDDLSAIA

NP31**

MTCHKLLATAGLLLIFAVWTSQAVNYYKQNKDTGSYSPPSSSRSRLPAKSQTGPSNALWIRTRDAPIDTLRYRVPMDRRMVPASMLFVDGNPDNMDKRLFEGLFPSNDRLNRYRRIAALLESEDDF*

NP32**

MAASVSFAFLMCIMLVTCALARPIGCQDSDRSSSPLARLMRRSAAIASRLRLLRSLRERRWARSYPIWKTLHRVQERNMDISHLPPLPLPDTEQHARDFDQIRAAVEKDSEGNKNSWVVGDVF EGGNWGSKPVVFSEVPNPEEENGGEVESDLNEGWDNGNPPIENEDNSDADWGEYKNPYLDCIDDNIVGIPCTRDSDCRGCTAYHCSASTNHCVAGPRPHMSFSGFI*

NP35**

MNSLCHVLILSAAFCLFLALTSGYPLQQAGNALREDLMERRSSPQRLTVDDLLDFLNERYQESPDKRQMRQTGLTAIDNVKGMYYNNNLNRMLAALQPHVRRR*

Fig. 1. Amino acid sequences of *H. leucospilota* neuropeptide precursors that have been characterised previously in other echinoderms. The predicted signal peptide is shown in bold letters with gray shading; cleavage sites are red underlined; cysteine residues are underlined with gray shading; glycine residues available for C-terminal amidation are with pink shading; *, indicates a full-length neuropeptide precursor. **, indicates a neuropeptide precursor that had not previously been found in sea cucumbers, and have now been identified in *H. leucospilota*.

Table 1
Holothuria leucospilota neuropeptides, including length, signal peptide size and percent similarity of precursor with neuropeptides of other sea cucumber species (if present). aa, indicates amino acid.

Neuropeptides	Length (aa)	Signal peptide size (aa)	% similarity *H. scabra*	% similarity *H. glaberrima*	% similarity *A. japonicus*	Neuropeptides	Length (aa)	Signal peptide size (aa)	% similarity *H. scabra*	% similarity *H. glaberrima*	% similarity *A. japonicus*
RGP	123	25	86.2	89.2	–	**NP11b**	94	20	79.8	93.1	–
Cubifrin	229	24	91.3	–	59.8	**NP15a**	111	19	43.1	–	–
GPB5-like 1	137	27	92.7	–	59.6	**NP15b**	105	20	80.9	93.1	–
GPB5-like 2	135	26	45.5	92.4	75.0	**NP18a**	113	27	27.5	–	36.3
Luqin-like	114	31	93.0	93.3	–	**NP18b**	121	25	96.7	95.4	72.5
Myomodulin-like	181	23	84.9	–	49.6	**NP21**	102	22	–	–	–
Corazonin-like 1	112	26	–	94.1	–	**NP23**	136	24	86.0	93.4	67.5
Corazonin-like 2	100	23	–	–	34.0	**NP25**	103	31	–	–	–
						NP26	75	21	–	–	–
PDF-type	102	22	–	88.6	–	**NP27**	102	20	–	–	–
CRH-type	132	28	–	–	–	**NP28**	104	29	–	–	–
Calcitonin-type	122	21	–	–	33.0	**NP30**	70	37	–	–	–
Orcokinin-type	373	31	–	20.7	–	**NP31**	126	23	–	–	–
Thyrostimulin2-like	127	24	60.8	66.5	56.8	**NP32**	229	21	–	–	–
Bursicon-like	139	34	29.4	–	31.4	**NP35**	103	23	–	–	–
						HleNP36	191	19	71.5	–	78.9
AN-type	205	24	–	36.3	–	**HleNP37**	165	27	95.8	–	50.6
TRH-like	138	16	96.0	90.9	65.1	**HleNP38**	113	22	94.9	97.7	–
Myoactive-like	138	25	59.8	85.7	60.0	**HleNP39**	92	22	56.5	80.3	43.5
Orexin-like	137	27	84.1	91.6	–	**HleNP40**	113	24	79.8	92.1	–
Holotocin-like	164	27	98.2	–	–	**HleNP41**	148	29	84.5	93.6	–
NP11a	102	22	26.8	–	–	**HleNP42**	121	33	86.0	97.0	–
						HleNP43	162	27	–	82.0	–

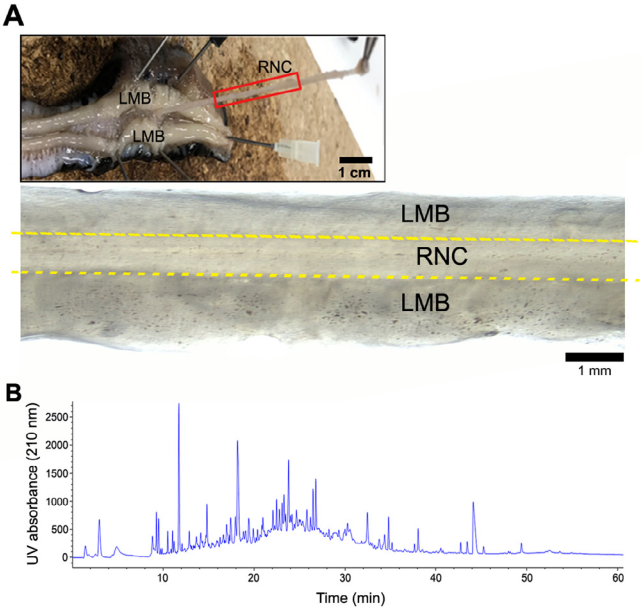

Fig. 2. Radial nerve cord of *H. leucospilota* and spectral profile of radial nerve extract (RNE). (A) Image of the dissected region of sea cucumber body wall from which a radial nerve cord (RNC) was separated. The picture below shows a higher magnification of the red box with RNC located between 2 longitudinal muscle bands (LMB). Yellow dashed lines indicate the boarders of RNC. (B) Representative reverse phase ultra-high performance liquid chromatography (RP-HPLC) chromatogram measuring UV absorbance (at 210 nm) of *H. leucospilota* RNE.

the absence of a stop codon (Fig. 1). Meanwhile, several neuropeptides were not found in *H. leucospilota* including GLRFA, glucoprotein hormone alpha-2, insulin-like growth factors, kisspeptin, stichopin, NP1, 2, 8, 9, 10, 13, 14, 16, 17, 19, 20, 22, 24, 29, 33 and 34, which have been identified in other echinoderms (Suwansa-ard et al., 2018).

Several neuropeptide precursors are present in multiple isoforms, including: glycoprotein hormone beta-5-like peptide (GPB5-like 1 and 2), corazonin-like peptide (corazonin-like 1 and 2), NP11 peptide (NP11a and 11b), NP15 peptide (NP15a and 15b), NP18 (NP18a and 18b) (Fig. 1). In neuropeptide precursors with two isoforms, GPB5-like 1, NP18b peptides of *H. leucospilota* showed a high conservation within *H. scabra*. The GPB5-like 2, corazonin-like 1, NP11b and NP15b are highly similar with peptides in *H. glaberrima* (Table 1). Meanwhile, corazonin-like 2, NP11a, NP15a and NP18a of *H. leucospilota* were conserved highly with corazonin-type, NP11, NP15 and NP18 of *A. rubens,* respectively (by BLASTp search in NCBI).

Of the 35 *H. leucospilota* neuropeptides, several are involved in echinoderm reproduction, such as RGP (relaxin-like gonad-stimulating peptide), cubifrin (NGIWYamide), myomodulin-like and CRH-type (corticotropin-releasing hormone-type) peptides. RGP was the first identified invertebrate gonadotropin to trigger final gamete maturation (Mita, 2016, 2013; Mita et al., 2009). Cubifrin has been reported as potent inducer of *in vitro* oocyte maturation and spawning in *A. japonicus* (Kato et al., 2009; Fujiwara et al., 2010b; Yamano et al., 2013) but is not effective in *H. leucospilota* (Chieu et al., 2018). Myomodulin has been implicated as an innervation messenger of male sexual system of snail *Lymnaea stagnalis* (De Lange et al., 1998; Koene, 2010). CRH-type/egg-laying hormone (ELH) is also suggested to be involved in reproductive control in molluscs (Zhang et al., 2018). However, the bioactive function of myomodulin-like and CRH-type in echinoderms remains to be investigated.

Echinoderms have several neuropeptides that can be categorised as myoactive peptides in function. Here we have identified *H. leucospilota* luqin-like, orcokinin-type, and myoactive-like (MPMNPADYFSRGTVYIPTRDS) precursor neuropeptides. Luqin-like causes dose-dependent relaxation of tube feet in *A. rubens* (Yañez-Guerra et al., 2018), while the orcokinin-type is the most potent muscle-relaxing peptide in the starfish *Patiria pectinifera* (Kim et al., 2018) and causes dose-dependent relaxation of cardiac stomach preparations in the starfish, *A. rubens* (Lin et al., 2018). The *A. japonicus* myoactive-like peptide (MPMNPADYFSRGTVYIPTRDS) has been also shown to induce contraction of the radial longitudinal muscles (Elphick, 2012).

Interestingly, we identified 11 neuropeptide precursors that had not previously been found in sea cucumbers, including a CRH-type, NP21, NP25, NP26, NP27, NP28, NP30, NP31, NP32, and NP35 peptide. They

Table 2
List of neuropeptide precursor-derived peptides detected by mass spectrometry in *H. leucospilota* radial nerve extract (RNE). *m/z*, mass to charge; AA, amino acid.

Protein Accession	Peptide sequence	Predicted average Mass (Da)	*m/z*	Charge	Precursor length AA	Start	End
HleNP36	G.IVAQGGVPFGGAGR.G	1284.6938	643.3613	2	191	122	135
HleNp37	R.SADSPYDAVAR.D	1150.5254	576.2747	2	165	107	117
HleNP38	R.TGGAC(+57.02)VYC(+57.02)R.R	1042.4324	522.2281	2	113	38	46
HleNP39	R.KLIYETC(+57.02)PK.N	1150.6056	576.3156	2	92	53	61
HleNP40	R.SALDDPSKR.D	987.4985	494.7608	2	113	40	48
HleNP41	R.RNNAC(+57.02)NPFTGK.C	1277.5935	639.8108	2	148	86	96
HleNP42	N.EDDDDALLR.H	1060.4673	531.2457	2	162	99	107
HleNP43	L.TPLTPR.K	683.3966	342.708	2	191	23	28

Fig. 3. Amino acid sequences of putative novel neuropeptide precursors in *H. leucospilota*. The predicted signal peptide is shown in bold letters with gray shading; cleavage sites are red underlined; cysteine residues are underlined with gray shading; glycine residues available for C-terminal amidation are with pink shading; *, indicates a full-length neuropeptide precursor. Blue bars show region of mass spectral peptide matches.

did not match with any protein, including neuropeptide, from other sea cucumber species (Table 1). The CRH-type peptides have been identified in various invertebrates such as insects (named diuretic hormone, DH44) (Cabrero et al., 2002), molluscs (egg-laying hormone, ELH) (Scheller et al., 1983; Conn and Kaczmarek, 1989; Cummins et al., 2001), annelids (Salzet et al., 1997) and starfish (Semmens et al., 2016). In this study, we found that the *H. leucospilota* CRH-type neuropeptide precursor was a 132-residue protein including a 28 residue signal peptide.

Other neuropeptides, named NP21, 25, 26, 27, 28, 30, 31, 32, and 35 have previously only been identified in the starfish *A. rubens* and *A. planci* (Semmens et al., 2016; Smith et al., 2017), although their function still remains unknown. As reported here, the homologs of these neuropeptides have now been found in *H. leucospilota*. Most of these neuropeptides contain two dibasic or monobasic cleavage sites at least, but only the partial-length peptide NP30 has no cleavage sites. Furthermore, NP25, 26, 27, 30, 32 contain two or more cysteine residues which creates the potential to establish disulphide cross-linkages that may be necessary for their bioactivity. In addition, NP21 and NP25 have glycine residues available for C-terminal amidation, a common post-translational modification that is essential for the biological activity of several peptide hormones (Merkler, 1994).

3.2. Putative novel neuropeptide precursors identified within the Holothuria leucospilota radial nerve cord

To further investigate the presence of neuropeptides in the sea cucumber, RNCs of adult *H. leucospilota* were isolated (Fig. 2A), then RNE

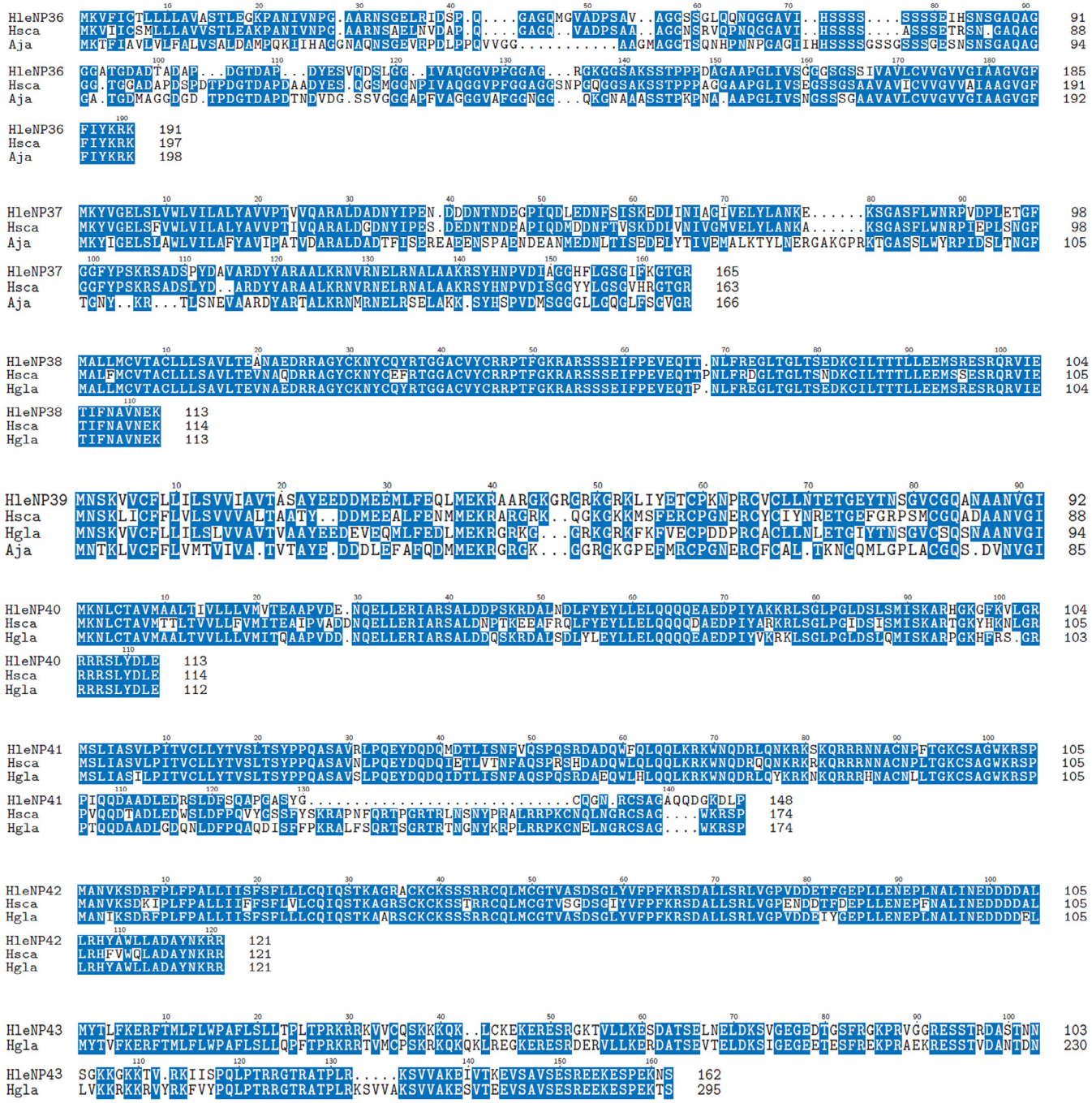

Fig. 4. Comparative sequence analysis of putative novel neuropeptide precursors in *H. leucospilota* with homologs in *Apostichopus japonicus, Holothura scabra*, and *H. glaberrima*. Hle, *Holothuria leucospilota;* Hsca, *Holothuria scabra;* Hgla, *Holothuria glaberrima;* Aja, *Apostichopus japonicus.* Blue shading indicates amino acid conservation. Information of percent similarity and conserved peptides is shown in Supplementary Data S1.

was processed for ultra-HPLC. Ultra-HPLC chromatograms showed the presence of numerous biomolecules (Fig. 2B), which were collected and analysed by MS. Eight novel peptides were identified and matched to precursors not previously characterized in other echinoderm species (Table 2). All contain features consistent with neuropeptide precursors, such as the presence of a signal peptide and dibasic cleavage sites, and they are full-length. These putative novel neuropeptides were named HleNP36 sequentially through to HleNP43 (Fig. 3, Genbank accession numbers MN159193, MN159194, MN159195, MN159196, MN159197, MN159198, MN159199, MN159200), following a system of nomenclature used previously for novel echinoderm neuropeptide precursors (Smith et al., 2017; Rowe and Elphick, 2012; Semmens et al., 2016). HleNP38, HleNP39, HleNP41 and HleNP42 each contain 4 cysteine residues, which may form intra-molecular disulfide bridges to establish quaternary protein structures required for bioactivity.

To investigate the existence of homolog precursors to these putative novel neuropeptide precursors in other echinoderms, a BLASTp search was implemented. Consequently, homologs with high conservation (*e*-value $<10^{-3}$) were found in other sea cucumber species, where genomic or transcriptome data is available (Fig. 4, Supplementary Data S1). No homologs were found in other echinoderm groups, suggesting that these putative neuropeptide precursors may be exclusive to sea cucumber species.

4. Conclusions

In this study, the total of 43 neuropeptide precursors (35 conserved and 8 novel) were identified in black sea cucumber *H. leucospilota* using a transcriptome resource established from its radial nerve cord, body wall and gonads. The 35 conserved neuropeptide precursors exhibit high homology with sea cucumber homologs. The 8 putative novel neuropeptide precursors appear to be exclusive to sea cucumbers, since they are absent from asteroid and echinoids. The knowledge obtained from this study will help with continuing efforts to characterize the function of echinoderm neuropeptides and to better understand eumetazoan neuropeptide evolution.

Acknowledgments

The authors gratefully acknowledge the financial support and laboratory facilities provided by the Genecology Research Centre, The Faculty of Science, Health, Education and Engineering and the Office of Research, USC, Australia. This research was supported by USC-VIED PhD scholarship (ID: 807E9516) to Hoang Dinh Chieu, which is funded by Ministry of Agriculture and Rural Development, Vietnamand USC, Australia. This research was undertaken with the assistance of the National Computational Infrastructure (NCI), which is supported by the Australian Government and the sequencing resources from Australian Genome Research Facility Ltd. (AGRF).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113229.

References

Burbach, J.P.H., 2011. What Are Neuropeptides? In: Merighi, A. (Ed.), Neuropeptides: Methods and Protocols. Humana Press, Totowa, NJ.
Burke, R.D., Angerer, L.M., Elphick, M.R., Humphrey, G.W., Yaguchi, S., Kiyama, T., Liang, S., Mu, X., Agca, C., Klein, W.H., Brandhorst, B.P., Rowe, M., Wilson, K., Churcher, A.M., Taylor, J.S., Chen, N., Murray, G., Wang, D., Mellott, D., Olinski, R., Hallbook, F., Thorndyke, M.C., 2006. A genomic view of the sea urchin nervous system. Dev. Biol. 300, 434–460.
Cabrero, P., Radford, J.C., Broderick, K.E., Costes, L., Veenstra, J.A., Spana, E.P., Davies, S.A., Dow, J.A.T., 2002. The Dh gene of *Drosophila melanogaster* encodes a diuretic peptide that acts through cyclic AMP. J. Exp. Biol. 205, 3799–3807.
Chieu, H.D., Suwansa-Ard, S., Abramov, T., Elizur, A., Cummins, S.F., 2018. *In vitro* oocyte maturation by radial nerve extract and early development of the black sea cucumber (*Holothuria leucospilota*). Aquaculture 495, 247–254.
Conn, P.J., Kaczmarek, L.K., 1989. The bag cell neurons of Aplysia. A model for the study of the molecular mechanisms involved in the control of prolonged animal behaviors. Mol. Neurobiol. 3, 237–273.
Cummins, S., Thongkukiatkul, A., Hanna, P.J., 2001. Location of egg-laying hormone in reproductive structures and neurons of *Haliotis rubra* (Leach) using antibodies raised against recombinant fusion proteins. J. Shellfish Res. 20, 705–710.
de Lange, R.P., Joosse, J., van Minnen, J., 1998. Multi-messenger innervation of the male sexual system of *Lymnaea stagnalis*. J. Comp. Neurol. 26, 564–577.
Drumm, D.J., Loneragan, N.R., 2005. Reproductive biology of *Holothuria leucospilota* in the Cook Islands and the implications of traditional fishing of gonads on the population. N. Z. J. Mar. Freshwater Res. 39, 141–156.
Elphick, M.R., 2012. The protein precursors of peptides that affect the mechanics of connective tissue and/or muscle in the echinoderm *Apostichopus japonicus*. PLoS ONE 7, 11.
Fahmy, S.R., Amer, M.A., Al-Killidar, M.H., 2015. Ameliorative effect of the sea cucumber *Holothuria arenicola* extract against gastric ulcer in rats. J. Basic Appl. Zool. 72, 16–25.
Fujiwara, A., Unuma, T., Ohno, K., Yamano, K., 2010a. Molecular characterization of the major yolk protein of the Japanese common sea cucumber (*Apostichopus japonicus*) and its expression profile during ovarian development. Comp. Biochem. Physiol. A-Mol. Integr. Physiol. 155, 34–40.
Fujiwara, A., Yamano, K., Ohno, K., Yoshikuni, M., 2010b. Spawning induced by cubifrin in the Japanese common sea cucumber *Apostichopus japonicus*. Fish. Sci. 76, 795–801.
Gaudron, S.M., Kohler, S.A., Conand, C., 2008. Reproduction of the sea cucumber *Holothuria leucospilota* in the Western Indian Ocean: biological and ecological aspects. Invertebr. Reprod. Dev. 51, 19–31.
Hökfelt, T., Broberger, C., Xu, Z.-Q.D., Sergeyev, V., Ubink, R., Diez, M., 2000. Neuropeptides — an overview. Neuropharmacology 39, 1337–1356.
John, H.B., Lynne, A.F., 2017. Neurotransmitters and Neuropeptides of Invertebrates. Oxford University Press.
Kato, S., Tsurumaru, S., Taga, M., Yamane, T., Shibata, Y., Ohno, K., Fujiwara, A., Yamano, K., Yoshikuni, M., 2009. Neuronal peptides induce oocyte maturation and gamete spawning of sea cucumber, *Apostichopus japonicus*. Dev. Biol. 326, 169–176.
Kim, C.H., Go, H.J., Oh, H.Y., Elphick, M.R., Park, N.G., 2018. Identification of evolutionarily conserved residues required for the bioactivity of a pedal peptide/orcokinin-type neuropeptide. Peptides 103, 10–18.
Koene, J.M., 2010. Neuro-endocrine control of reproduction in hermaphroditic freshwater snails: mechanisms and evolution. Front. Behav. Neurosci. 4, 167.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
Lin, M., Egertova, M., Zampronio, C.G., Jones, A.M., Elphick, M.R., 2018. Functional characterization of a second pedal peptide/orcokinin-type neuropeptide signaling system in the starfish *Asterias rubens*. J. Comp. Neurol. 526, 858–876.
Lovatelli, A., 2004. Advances in sea cucumber aquaculture and management. FAO 463, 440.
Mashanov, V.S., Zueva, O.R., Garcia-Arraras, J.E., 2014. Transcriptomic changes during regeneration of the central nervous system in an echinoderm. Bmc Genomics 15.
Merkler, D.J., 1994. C-terminal amidated peptides: production by the in vitro enzymatic amidation of glycine-extended peptides and the importance of the amide to bioactivity. Enzyme Microb. Technol. 16, 450–456.
Mita, M., 2013. Relaxin-like gonad-stimulating substance in an echinoderm, the starfish: a novel relaxin system in reproduction of invertebrates. Gen. Comp. Endocrinol. 181, 241–245.
Mita, M., 2016. Starfish gonadotropic hormone: relaxin-like gonad-stimulating peptides. Gen. Comp. Endocrinol. 230–231, 166–169.
Mita, M., Yoshikuni, M., Ohno, K., Shibata, Y., Paul-Prasanth, B., Pitchayawasin, S., Isobe, M., Nagahama, Y., 2009. A relaxin-like peptide purified from radial nerves induces oocyte maturation and ovulation in the starfish, *Asterina pectinifera*. PNAS 106, 9507–9512.
Morgan, A., Neal, L., 2012. Aspects of reproductive ecology and benthic–pelagic coupling in the sub-antarctic sea cucumber *Pseudostichopus mollis* (Theel). Cont. Shelf Res. 43, 36–42.
Nahla, E.S.E.S.O., 2013. Nutritional value of some Egyptian sea cucumbers. Afr. J. Biotechnol. 12, 5466–5472.
Nässel, D.R., Larhammar, D., 2013. Neuropeptides and peptide hormones. In: Galizia, C.G., Lledo, P.-M. (Eds.), Neurosciences – From Molecule to Behavior: A University Textbook. Springer Berlin Heidelberg, Berlin, Heidelberg.
Ni, G., Liang, D., Cummins, S.F., Walton, S.F., Chen, S., Wang, Y., Mounsey, K., Wei, M.Q., Yuan, J., Pan, X., Liu, X., Wang, T., 2018. Comparative Proteomic Study of the Antiproliferative Activity of Frog Host-Defence Peptide Caerin 1.9 and Its Additive Effect with Caerin 1.1 on TC-1 Cells Transformed with HPV16 E6 and E7. Biomed Res. Int. 2018, 7382351.
Olivera-Castillo, L., Davalos, A., Grant, G., Valadez-Gonzalez, N., Montero, J., Barrera-Perez, H.A.M., Chim-Chi, Y., Olvera-Novoa, M.A., Ceja-Moreno, V., Acereto-Escoffie, P., Rubio-Piña, J., Rodriguez-Canul, R., 2013. Diets Containing Sea Cucumber (*Isostichopus badionotus*) Meals Are Hypocholesterolemic in Young Rats. PLoS ONE 8, e79446.
Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785.
Purcell, S.W., Mercier, A., Conand, C., Hamel, J.-F., Toral-Granda, M.V., Lovatelli, A., Uthicke, S., 2011. Sea cucumber fisheries: global analysis of stocks, management measures and drivers of overfishing. Fish Fish. 14, 34–59.
Rowe, M.L., Achhala, S., Elphick, M.R., 2014. Neuropeptides and polypeptide hormones in echinoderms: new insights from analysis of the transcriptome of the sea cucumber *Apostichopus japonicus*. Gen. Comp. Endocrinol. 197, 43–55.
Rowe, M.L., Elphick, M.R., 2012. The neuropeptide transcriptome of a model echinoderm, the sea urchin *Strongylocentrotus purpuratus*. Gen. Comp. Endocrinol. 179, 331–344.
Salzet, M., Verger-Bocquet, M., Vandenbulcke, F., van Minnen, J., 1997. Leech egg-laying-like hormone: structure, neuronal distribution and phylogeny. Brain Res. Mol. Brain Res. 49, 211–221.
Scheller, R.H., Jackson, J.F., Mcallister, L.B., Rothman, B.S., Mayeri, E., Axel, R., 1983. A single gene encodes multiple neuropeptides mediating a stereotyped behavior. Cell 32, 7–22.
Semmens, D.C., Mirabeau, O., Moghul, I., Pancholi, M.R., Wurm, Y., Elphick, M.R., 2016. Transcriptomic identification of starfish neuropeptide precursors yields new insights into neuropeptide evolution. Open Biol. 6.
Smith, M.K., Wang, T.F., Suwansa-Ard, S., Motti, C.A., Elizur, A., Zhao, M., Rowe, M.L., Hall, M.R., Elphick, M.R., Cummins, S.F., 2017. The neuropeptidome of the Crown-of-Thorns Starfish, *Acanthaster planci*. J. Proteomics 165, 61–68.
Sodergren, E., Weinstock, G.M., Davidson, E.H., Cameron, R.A., Gibbs, R.A., Angerer, R.C., Angerer, L.M., Arnone, M.I., Burgess, D.R., Burke, R.D., Coffman, J.A., Dean, M., Elphick, M.R., Ettensohn, C.A., Foltz, K.R., Hamdoun, A., Hynes, R.O., Klein, W.H., Marzluff, W., McClay, D.R., Morris, R.L., Mushegian, A., Rast, J.P., Smith, L.C., Thorndyke, M.C., Vacquier, V.D., Wessel, G.M., Wray, G., Zhang, L., Elsik, C.G., Ermolaeva, O., Hlavina, W., Hofmann, G., Kitts, P., Landrum, M.J., Mackey, A.J., Maglott, D., Panopoulou, G., Poustka, A.J., Pruitt, K., Sapojnikov, V., Song, X., Souvorov, A., Solovyev, V., Wei, Z., Whittaker, C.A., Worley, K., Durbin, K.J., Shen, Y., Fedrigo, O., Garfield, D., Haygood, R., Primus, A., Satija, R., Severson, T., Gonzalez-Garay, M.L., Jackson, A.R., Milosavljevic, A., Tong, M., Killian, C.E., Livingston, B.T., Wilt, F.H., Adams, N., Bellé, R., Carbonneau, S., Cheung, R., Cormier, P., Cosson, B., Croce, J., Fernandez-Guerra, A., Geneviève, A.-M., Goel, M., Kelkar, H., Morales, J., Mulner-Lorillon, O., Robertson, A.J., Goldstone, J.V., Cole, B., Epel, D., Gold, B., Hahn, M.E., Howard-Ashby, M., Scally, M., Stegeman, J.J., Allgood, E.L., Cool, J., Judkins, K.M., McCafferty, S.S., Musante, A.M., Obar, R.A., Rawson, A.P., Rossetti, B.J., Gibbons, I.R., Hoffman, M.P., Leone, A., Istrail, S.,

Materna, S.C., Samanta, M.P., Stolc, V., Tongprasit, W., et al., 2006. The Genome of the Sea Urchin *Strongylocentrotus purpuratus*. Science 314, 941.

Southey, B.R., Rodriguez-Zas, S.L., Sweedler, J.V., 2009. Characterization of the prohormone complement in cattle using genomic libraries and cleavage prediction approaches. BMC Genom. 10 (228), 1–11.

Suwansa-Ard, S., Chaiyamoon, A., Talarovicova, A., Tinikul, R., Tinikul, Y., Poomtong, T., Elphick, M.R., Cummins, S.F., Sobhon, P., 2018. Transcriptomic discovery and comparative analysis of neuropeptide precursors in sea cucumbers (Holothuroidea). Peptides 99, 231–240.

Yamano, K., Fujiwara, A., Nakamura, A., Yoshikuni, M., 2013. *In vitro* induction of oocyte maturation in the Japanese sea cucumber *Apostichopus japonicus* by cubifrin and the developmental ability of the eggs. Fish. Sci. 79, 823–832.

Yañez-Guerra, L.A., Delroisse, J., Barreiro-Iglesias, A., Slade, S.E., Scrivens, J.H., Elphick, M.R., 2018. Discovery and functional characterisation of a luqin-type neuropeptide signalling system in a deuterostome. Sci. Rep. 8, 7220.

Zhang, M., Wang, Y., Li, Y., Li, W., Li, R., Xie, X., Wang, S., Hu, X., Zhang, L., Bao, Z., 2018. Identification and Characterization of Neuropeptides by Transcriptome and Proteome Analyses in a Bivalve Mollusc *Patinopecten yessoensis*. Front. Genet. 9 197 197.

Zhang, X.J., Sun, L.N., Yuan, J.B., Sun, Y.M., Gao, Y., Zhang, L.B., Li, S.H., Dai, H., Hamel, J.F., Liu, C.Z., Yu, Y., Liu, S.L., Lin, W.C., Guo, K.M., Jin, S.J., Xu, P., Storey, K.B., Huan, P., Zhang, T., Zhou, Y., Zhang, J.Q., Lin, C.G., Li, X.N., Xing, L.L., Huo, D., Sun, M.Z., Wang, L., Mercier, A., Li, F.H., Yang, H.S., Xiang, J.H., 2017. The sea cucumber genome provides insights into morphological evolution and visceral regeneration. PLoS Biol. 15, 31.